Anal. Chem. **2000**, *72*, 3007–3013

Accelerated Articles

Liquid Chromatography/Electron Capture Atmospheric Pressure Chemical Ionization/Mass Spectrometry: Analysis of Pentafluorobenzyl Derivatives of Biomolecules and Drugs in the Attomole Range

Gurkeerat Singh,† Alejandro Gutierrez, Keyang Xu,‡ and Ian A. Blair*

Center for Cancer Pharmacology, University of Pennsylvania, 421 Curie Boulevard, Philadelphia, Pennsylvania 19104-6160

The corona discharge used to generate positive and negative ions under conventional atmospheric pressure chemical ionization conditions also provides a source of gas-phase electrons. This is thought to occur by displacement of electrons from the nitrogen sheath gas. Therefore, suitable analytes can undergo electron capture in the gas phase in a manner similar to that observed for gas chromatography/electron capture negative chemical ionization/mass spectrometry. This technique, which has been named electron capture atmospheric pressure chemical ionization/mass spectrometry, provided an increase in sensitivity of 2 orders of magnitude when compared with conventional atmospheric pressure chemical ionization methodology. It is a simple procedure to tag many biomolecules and drugs with an electron-capturing group such as the pentafluorobenzyl moiety before analysis. Pentafluorobenzyl derivatives have previously been used as electron capturing derivatives because they undergo dissociative electron capture in the gas phase to generate negative ions through the loss of a pentafluorobenzyl radical. A similar process was found to occur under electron capture atmospheric pressure chemical ionization conditions. By monitoring the negative ions that were formed, it was possible to obtain attomole sensitivity for pentafluorobenzyl derivatives of a representative steroid, steroid metabolite, prostaglandin, thromboxane, amino acid, and DNA–adduct.

Liquid chromatography (LC) together with atmospheric pressure ionization (API)-based mass spectrometry (MS) methodology has revolutionized our approach to the analysis of biomolecules[1-3] and drugs.[4,5] LC/atmospheric pressure chemical ionization (APCI)/MS is a useful API technique because analyte signals are relatively insensitive to suppression by contaminants from the biological matrix.[6-8] Therefore, this technique is often used for accurate and precise analyses of biomolecules, drugs, and their metabolites in biological fluids. Unfortunately, LC/APCI/MS methodology does not have the sensitivity to compete with gas chromatography/electron capture negative chemical ionization (GC/ECNCI)/MS, which was introduced by Hunt and co-workers in 1976.[9] Therefore, GC/ECNCI/MS, which is a time-consuming and complex technique, is still the method of choice for the trace analysis of many

* To whom correspondence should be addressed: 1254 BRB II/III; (phone) (215) 573-9885; (fax) (215) 573-9889; (e-mail) ian@spirit.gcrc.upenn.edu.

† Current address: Lilly Research Laboratories, Eli Lilly and Co., Lilly Corporate Center, Indianapolis, IN 46285.

‡ Current address: Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320-1799.

(1) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64–71.

(2) Hunt, D. F.; Henderson, R. A.; Shabonowitz, J.; Sakaguchi, K.; Michel, H.; Sevilir, N.; Cox, A. L.; Appella, E.; Engelhard, V. H. *Science* **1992**, *255*, 1261–6.

(3) Siuzdak, G. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11290–7.

(4) Blair, I. A. In *Pharmacokinetics of Drugs: Handbook of Experimental Pharmacology*; Welling, P. G., Balant, L. P., Eds.; Springer-Verlag GmbH & Co.: Heidelberg, 1994.

(5) Brewer, E.; Henion, J. *J. Pharm. Sci.* **1998**, *87*, 395–402.

(6) Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. *Anal. Chem.* **1998**, *70*, 882–9.

(7) Singh, G.; Arora, V. K.; Fenn, P. T.; Mets, B.; Blair, I. A. *Anal. Chem.* **1999**, *71*, 2021–7.

(8) Schaefer, W. H.; Dixon, F., Jr. *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 1059–69.

(9) Hunt, D. F.; Stafford, G. C., Jr.; Crow, F. W.; Russell, J. W. *Anal. Chem.* **1976**, *48*, 2098–105.

10.1021/ac000374a CCC: $19.00
Published on Web 06/09/2000

important biomolecules, drugs, and their metabolites.[4,10-12] The pioneering work of Horning et al. showed that ionization under APCI conditions was initiated by the $N_2^{\cdot+}$ radical cation, which in turn was formed by collision of high-energy electrons from the corona discharge with the nitrogen sheath gas.[13,14] However, the potential analytical utility of the low-energy electrons that also result from these collisions has not been recognized. It is conceivable that the low-energy electrons generated in the APCI source could potentially ionize suitable analytes through dissociative electron capture. The ionization process would then be analogous to ECNCI in a conventional chemical ionization source.[9] We report the finding that electron capture APCI (ECAPCI) can indeed be observed in the APCI source and that ultrahigh sensitivity can be obtained with suitable electron-capturing derivatives. We also describe the potential utility of the technique for the analysis of steroids, prostaglandins, and DNA−adducts in the attomole range.

EXPERIMENTAL SECTION

Apparatus. LC was performed on a Waters 2690 separation module equipped with an autosampler, a vacuum degasser, and a column heater (Waters, Milford, MA). This was coupled to a Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer fitted with an APCI source (Finnigan Corp., San Jose, CA). The YMCbasic, YMC ODS-AQ, and silica columns were purchased from Waters. Evaporations under nitrogen were conducted using an N-evap analytical evaporator (Organomation, Berlin, MA). Precolumn filters (2 μm) were from Alltech, Deerfield, IL.

Reagents. Estrone, estradiol, 16α-hydroxyestrone, and 2-methoxyestrone were obtained from Steraloids Inc. (Newport, RI). [$^2H_4$]-estrone and [$^2H_3$]estradiol were obtained from CDN Isotopes (Quebec, Canada). Prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) and 11-dehydrothromboxane B_2 (11-dehydro-TXB_2) were obtained from Cayman Chemical Co. (Ann Arbor, MI). Phenylalanine, diisopropylethylamine, and pentafluorobenzyl bromide (α-bromo-2,3,4,5,6-pentafluorotoluene) were obtained from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade water, Optima grade acetonitrile, methanol, and hexane were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Ethanol was from Pharmco (Brookfield, CT). Ammonium acetate was obtained from J. T. Baker (Phillipsburg, NJ). The heptanone etheno-2′-deoxyguanosine adduct was synthesized in our laboratory.[15]

Derivatization. Estrone (100 ng, 370 pmol), 2-methoxyestrone (100 ng, 333 pmol), 16α-hydroxyestrone (100 ng, 350 pmol), [$^2H_4$]-estrone (100 ng, 365 pmol), estradiol (100 ng, 368 pmol), [$^2H_3$]-estradiol (100 ng, 364 pmol), 11-dehydro-TXB_2 (100 ng, 272 pmol), phenylalanine (100 ng, 290 pmol), or heptanone etheno-2′-deoxyguanosine (100 μg, 248 nmol) in acetonitrile (50 μL) was treated with 50 μL of PFB bromide in acetonitrile (1:19; v/v) followed by 50 μL of potassium hydroxide in anhydrous ethanol (8:1000; w/v), and the solution was heated at 60 °C for 30 min. The solution was allowed to cool, evaporated to dryness under nitrogen at room temperature, and redissolved in methanol/water (7:3; v/v) for reversed-phase conditions or hexane/ethanol (97:3: v/v) for normal-phase chromatography ready for LC/MS analysis. $PGF_{1\alpha}$ (100 ng, 281 pmol) in acetonitrile (50 μL) was treated with 50 μL of PFB bromide in acetonitrile (1:19; v/v) followed by 50 μL of diisopropylethylamine in acetonitrile (1:9; v/v). The solution was heated at 65 °C for 60 min, allowed to cool, evaporated to dryness under nitrogen at room temperature, and redissolved in methanol/water (7:3) ready for LC/MS analysis.

Mass Spectrometry. The mass spectrometer operating conditions were as follows: vaporizer temperature 475 °C, heated capillary at 300 °C, with the corona discharge needle set at 16 μA. The sheath gas (nitrogen) and auxiliary gas (nitrogen) pressures were set at 34 psi and 10 (arbitrary units), respectively. collision-induced dissociation (CID) was performed using argon as the collision gas at 2.9 mTorr in the second (rf-only) quadrupole. For full-scan and selected reaction monitoring (SRM) analyses, unit resolution was maintained for both parent and product ions. The method was robust; any minor deposits on the corona discharge needle were removed each morning after operating for 24 h.

Liquid Chromatography. The volume for all sample injections on the LC was 10 μL. Gradient elutions were performed in the linear mode. Systems 1−4 used a YMCbasic column (150 × 4.6 mm, i.d., 5 μm) at a flow rate of 1.0 mL/min. Solvent A was water and solvent B was methanol. The column was allowed to equilibrate for 5 min after each gradient elution. The gradient for system 1 was 70% B for 2.5 min, followed by a gradient to 100% B over 4.5 min, which was held at 100% B for 4 min before returning to starting conditions over 2 min. The gradient for system 2 was 70% B for 2.0 min, followed by a gradient to 100% B over 14.0 min, which was at 100% B for 1 min before returning to starting conditions over 3 min. The gradient for system 3 was 75% B for 1.0 min, followed by a gradient to 100% B over 1.5 min, which was held at 100% B for 2.5 min before returning to starting conditions over 1 min. The gradient for system 4 was 35% B for 1.5 min, followed by a gradient to 100% B over 4.5 min, which was held at 100% B for 5 min before returning to starting conditions over 3 min. Chromatography system 5 employed a YMC ODS-AQ column (150 × 2.0 mm, i.d.; 5 μm) at a mobile-phase flow rate of 0.2 mL/min with a postcolumn addition of 0.8 mL/min methanol. Solvent A was water and solvent B was acetonitrile. Gradient elution for system 5 was as follows: a gradient was run from 25% to 65% B over 8 min, followed by a second gradient to 100% B over 2 min before returning to the starting conditions over 4 min. Chromatography system 6 used a normal-phase isocratic mobile phase of hexane/ethanol (97:3; v/v). Chromatography was conducted on a silica column (150 × 2 mm i.d.; 120 Å, S-5) at a flow rate of 0.2 mL/min with a postcolumn addition of methanol (0. 8 mL/min).

LC/ECAPCI Analysis of Estrogens. LC/SRM/MS was conducted using 50 pg of each estrogen as its PFB derivative. The following SRM transitions were monitored: 16α-hydroxyestrone-PFB m/z 285 → 145 (collision energy 45 eV), 2-methoxyestrone-PFB m/z 299 → 284 (collision energy 24 eV), estrone-PFB m/z 269 → 145 (collision energy 40 eV), [$^2H_4$]estrone-PFB,

(10) Blair, I. A. In *Methods in enzymology*, 187th ed.; Murphy, R. C., Fitzpatrick, F. A., Eds.; Academic Press: Orlando, FL, 1990.
(11) Blair, I. A. *Chem. Res. Toxicol.* **1993**, *6*, 741−7.
(12) Giese, R. W. *Chem. Res. Toxicol.* **1997**, *10*, 255−70.
(13) Horning, E. C.; Carroll, D. I.; Dzidic, I.; Haegele, K. D.; Horning, M. G.; Stillwell, R. N. *J. Chromatogr. Sci.* **1974**, *12*, 725−9.
(14) Carroll, D. I.; Dzidic, I.; Stillwell, R. N.; Haegele, K. D.; Horning, E. C. *Anal. Chem.* **1975**, *47*, 2369−73.
(15) Rindgen, D.; Nakajima, M.; Wehrli, S.; Xu, K.; Blair, I. A. *Chem. Res. Toxicol.* **1999**, *12*, 1195−204.

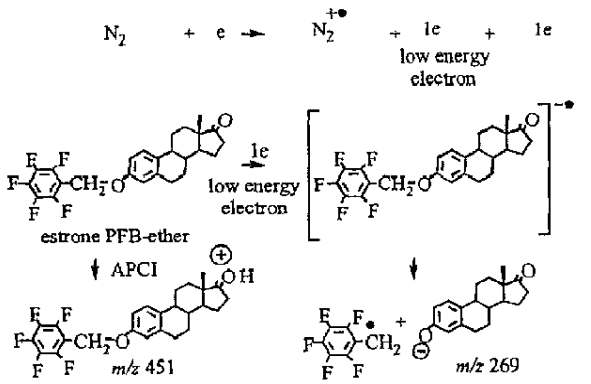

Figure 1. Proposed mechanism for ECAPCI/MS. Electrons generated from the corona discharge interact with nitrogen, which is used as the sheath gas, to generate radical cation and low-energy thermal electrons. These low-energy thermal electrons interact with estrone PFB-ether in the APCI source. Dissociative electron capture would result in the formation of an intense negative ion for estrone at *m/z* 269. Under conventional positive APCI conditions, a protonated molecular ion would be observed at *m/z* 451.

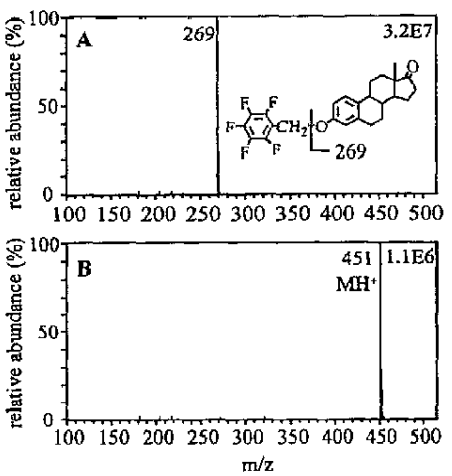

Figure 2. (A) ECAPCI mass spectrum of estrone-PFB. (B) Positive APCI mass spectrum of estrone-PFB.

m/z 273 → 147 (collision energy 41 eV), estradiol-PFB *m/z* 271 → 183 (collision energy 52 eV), and [$^2H_3$]estradiol-PFB *m/z* 274 → 185 (collision energy 48 eV).

RESULTS AND DISCUSSION

LC/ECAPCI/MS. If electron capture could occur in the APCI source, suitable analytes would undergo dissociative electron capture. This is illustrated in Figure 1 for the PFB ether derivative of estrone. The ionization process would then be analogous to ECNCI in a conventional chemical ionization source.[9] When estrone-PFB was analyzed under negative APCI conditions, an intense ion was observed at *m/z* 269 (Figure 2A), corresponding to the loss of a PFB radical from the molecular ion $[M]^{-\cdot}$. If conventional APCI had occurred, then an $[M - H]^-$ ion would have been observed at *m/z* 449. In fact, when estrone-PFB ether was analyzed by positive APCI, the expected mass spectrum was obtained in which MH^+ was observed as a major ion at *m/z* 451

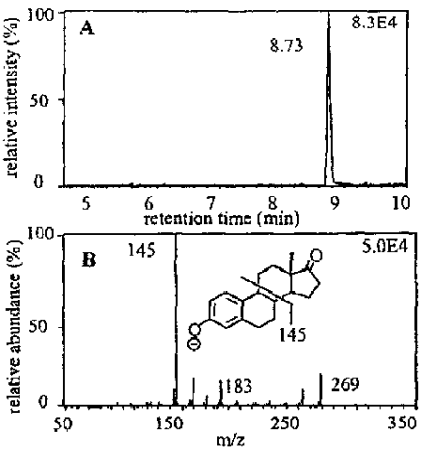

Figure 3. (A) LC/ECAPCI/MS chromatogram of 10 pg of estrone on column with a retention time of 8.73 min. Chromatography was performed using system 1. (B) Product ion spectra from CID of $[M - PFB]^-$ ion *m/z* 269 at a collision energy of 44 eV.

(Figure 2B). Therefore, under negative APCI conditions, dissociative electron capture had indeed occurred to form an intermediate radical anion, which after loss of the PFB radical produced an intense $[M - PFB]^-$ negative ion at *m/z* 269 (Figure 1). Importantly, the structural integrity of the steroid molecule was maintained during the electron capture process. These data convincingly demonstrated that a novel ionization process had occurred, which we have termed ECAPCI.

Analysis of Estrone-PFB by LC/ECAPCI/MS. Analysis of estrone-PFB by LC/ECAPCI/MS using gradient system 1 revealed that the derivative had excellent chromatographic properties under reversed-phase conditions (Figure 3A). The mass spectrum obtained by CID and MS/MS analysis of $[M - PFB]^-$ from estrone-PFB (*m/z* 269) is shown in Figure 3B. The product ion spectrum was essentially identical with that observed from underivatized estrone. A major product ion was observed at *m/z* 145, which resulted from fragmentation in the C-ring of the steroid molecule. SRM was then performed on the transition *m/z* 269 → 145. To determine the limit of detection for estrone as its PFB derivative, a blank injection was first performed monitoring the transition *m/z* 269 → 145. No signal was observed at the retention time of estrone-PFB (Figure 4A upper). A sample of 200 fg (740 amol) of estrone as its PFB was then analyzed. A response was observed at the retention time for estrone-PFB with a signal/noise ratio (S/N) of >5:1 (Figure 4A lower). The response was linear over at least 3 orders of magnitude (Figure 4B).

Analysis of Estrogen-PFB Derivatives by Reversed-Phase LC/ECAPCI/MS. One of the advantages of LC/MS over other high-sensitivity techniques (such as immunoassays) is the ability to perform quantitation of more than one compound in a single analytical procedure. We have tested the utility of LC/ECAPCI/MS for multicompound determinations by analyzing a mixture of estradiol and its metabolites. These biomolecules were chosen because of our interest in the potential role of estrogen metabolism in carcinogenesis[16,17] and because estrogens represent one of the

(16) Service, R. F. *Science* **1998**, *279*, 1631–3.
(17) Zhu, B. T.; Conney, A. H. *Cancer Res.* **1998**, *58*, 2269–77.

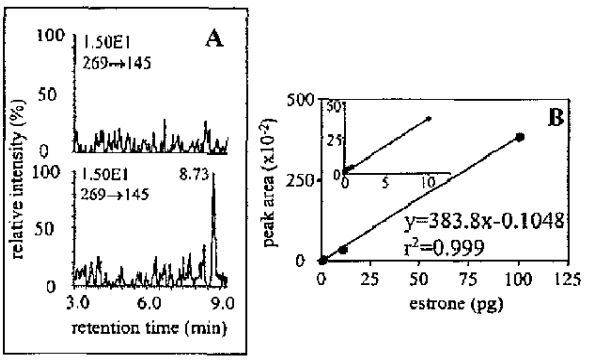

Figure 4. (A) Limit of detection determination of estrone. Top panel: 10 μL of solvent blank injected preceding to the LOD determination. Bottom panel: 200 fg (740 amol) of estrone on column with signal-to-noise ratio of 6:1. Chromatography was performed using system 1. (B) Standard curve for the analysis of estrone in the range of 200 fg to 100 pg. The insert shows the linear range of 200 fg to 10 pg.

most widely prescribed categories of drugs.[18] Ultrahigh sensitivity detection is necessary because of the very low concentrations of plasma estrogens.[19] PFB derivatives of 16α-hydroxyestrone, 2-methoxyestrone, estrone, and estradiol were formed in essentially quantitative yield. [$^2H_4$]Estrone and [$^2H_3$]estradiol were included in the steroid mixture because they will be employed as internal standards in future quantitative assays. SRM analyses were performed using the transition from [M − PFB] to a major product ion. Under reversed-phase conditions using gradient system 2, complete separation was accomplished between all of the steroids within a 13-min run time (Figure 5). The estrogen PFB derivatives were detected with high sensitivity. The signal from 2-methoxyestrone was particularly impressive; an injection of 50 fg (170 amol) was readily detected (Table 1).

Analysis of Estrogen-PFB Derivatives by Normal -Phase LC/ECAPCI/MS. One of the potential advantages of ECAPCI/MS is the ability to employ normal-phase solvents. This would greatly extend the range of available stationary phases by which ultrahigh sensitivity analyses could be conducted. In particular, it would allow isomeric analytes such as prostaglandins and hydroxyeicosatetraenoic acids that are poorly separated under reversed-phase conditions to be readily separated. Furthermore, it would make it possible to use a wide range of chiral columns that are normally restricted to normal-phase solvents. The ability to analyze PFB derivatives under normal-phase conditions was tested using the same estrogen mixture that was employed for reversed-phase chromatography. Using the hexane/ethanol LC system 6, it was possible to completely separate the estrogen-PFB derivatives in a manner analogous to that accomplished under reversed-phase conditions (Figure 6). The sensitivity with which these analyses were accomplished was almost identical. Although, a postcolumn addition of methanol was employed in this experiment, subsequent studies have shown that this is unnecessary. The limits of detection using normal-phase solvents are essentially identical with those observed for reversed-phase conditions.

(18) Shen, L.; Qui, S.; Chen, Y.; Zhang, F.; van Breemen, R. B.; Nikolic, D.; Bolton, J. L. *Chem. Res. Toxicol.* **1998**, *11*, 94−101.

(19) Matsuhashi, N.; Nakajima, A.; Fukushima, Y.; Yazaki, Y.; Oka, T. *Gut* **1997**, *40*, 344−9.

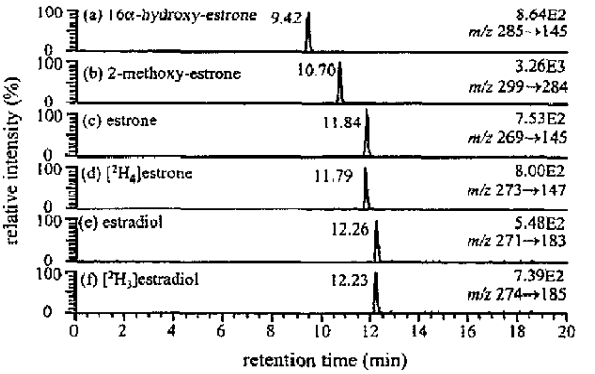

Figure 5. Reversed-phase separation of PFB derivatives of estradiol (50 pg) and its metabolites (50 pg of each) by LC/ECAPCI/SRM/MS using chromatography system 2. (a) 16α-hydroxyestrone-PFB, retention time 9.42 min, monitoring the transition *m/z* 285→ 145 at a collision energy of 45 eV; (b) 2-methoxyestrone-PFB, retention time 10.70 min, monitoring the transition *m/z* 299→ 284 at a collision energy of 24 eV; (c) estrone-PFB, retention time 11.84 min, monitoring the transition *m/z* 269→ 145 at a collision energy of 40 eV; (d) [$^2H_4$]-estrone-PFB, retention time 11.79 min, monitoring the transition *m/z* 273→ 147 at a collision energy of 41 eV; (e) estradiol-PFB, retention time 12.26 min, monitoring the transition *m/z* 271→ 183 at collision energy of 52 eV; and (f) [$^2H_3$]estradiol-PFB, retention time 12.23 min, monitoring the transition *m/z* 274→ 185 at collision energy of 48 eV.

Table 1. Limits of Detection Using APCI and ECAPCI for Selected Analytes

analyte	negative APCI[a] LOD (fmol)	ECAPCI[b] LOD (amol)	increased sensitivity[c]
estrone	18.52	740	25-fold
2-methoxyestrone	8.33	170	50-fold
prostaglandin $F_{1\alpha}$	7.02	140	50-fold
11-dehydro-TXB_2	54.35	540	100-fold
phenylalanine	60.61	610	100-fold
hepatanone ϵ-dGuo	62.03	710	100-fold

[a] Underivatized analyte. [b] PFB derivative. [c] Negative ion APCI of underivatized analyte compared with ECAPCI of PFB derivative.

Analysis of DNA Adduct PFB Derivatives by LC/ECAPCI/MS. Another area where there is a compelling need for high sensitivity is in the analysis of DNA−adducts. Currently, GC/ECNCI/MS is the method of choice because of its exquisite sensitivity.[12] Unfortunately, it is first necessary to remove the 2′-deoxyribose moiety prior to the analysis of DNA adducts because of its poor volatility. This is particularly problematic for urinary DNA adducts because it becomes impossible to determine whether an adduct is derived from DNA or RNA. We have recently demonstrated that lipid hydroperoxides undergo a specific homolytic decomposition to generate 4-oxo-2-nonenal.[15] This novel bifunctional electrophile efficiently forms a substituted etheno (ϵ) adduct with 2′-deoxyguanosine (dGuo) similar to that obtained with 4-oxo-2-pentenal.[20] We required a highly sensitive method for analysis of the heptanone ϵ-dGuo adduct in order to determine whether it was formed in vivo. Our previous studies have demonstrated that malondialdehyde (another product of lipid peroxidation) can form an adduct with dGuo (M_1G) in vivo.[21,22]

(20) Liu, Z.; Young-Sciame, R.; Hecht, S. S. *Chem. Res. Toxicol.* **1996**, *9*, 774−80.

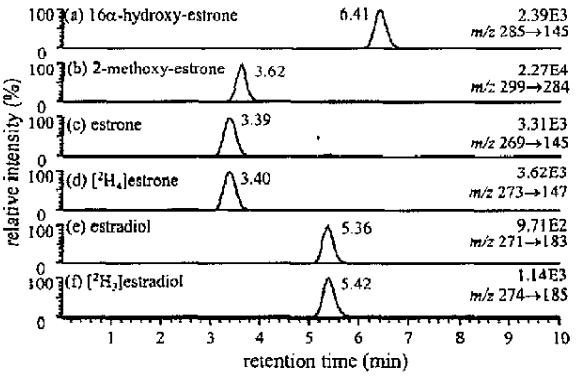

Figure 6. Normal-phase separation of PFB derivatives of estradiol and its metabolites by LC/ECAPCI/SRM/MS using chromatography system 6. MS conditions were the same as described for Figure 5.

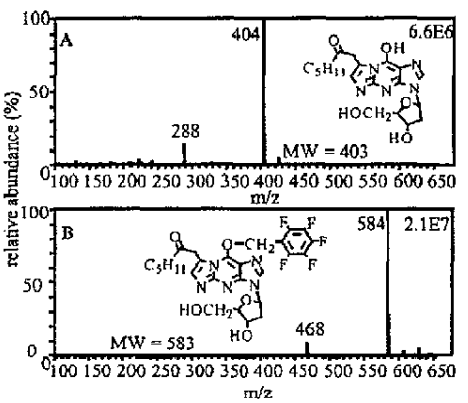

Figure 7. (A) Positive APCI mass spectrum of heptanone ϵ-dGuo. (B) Positive APCI mass spectrum of heptanone ϵ-dGuo-PFB.

The ability to monitor both M_1G and the substituted ϵ-dGuo in vivo would provide a powerful molecular dosimeter with which to elucidate the role of lipid peroxidation-mediated damage to DNA in carcinogenesis. LC/MS analysis in the positive APCI mode showed the expected MH^+ ions for heptanone ϵ-dGuo (Figure 7A) and its PFB derivative (Figure 7B) at *m/z* 404 and 584, respectively. However, under negative APCI conditions, both heptanone ϵ-dGuo (Figure 8A) and its PFB derivative (Figure 8B) showed the same intense negative ion at *m/z* 402. This provided clear evidence that the PFB moiety was lost under negative ion conditions and that dissociative electron capture had occurred. CID and MS/MS analysis of *m/z* 402 revealed two major product ions at *m/z* 286 and 188 (Figure 9A). LC/MS analysis under negative ion conditions revealed that the PFB derivative had excellent LC properties (Figure 9B). LC/SRM/MS analysis of the specific transition *m/z* 402 → 286 using gradient system 5 provided a limit of detection of 100 fg (710 amol) on column (Table 1). A linear response was obtained when increasing amounts of the heptanone ϵ-dGuo-PFB derivative (100 fg to 10 pg) were analyzed (Figure 9C).

Analysis of Prostaglandin-, Thromboxane-, and Phenylalanine-PFB Derivatives by LC/ECAPCI/MS. There are numerous other important potential applications of the ECAPCI/MS methodology. Three areas that will be of immediate utility will be for PGs, TXs, and amino acids. To date, there has been a paucity of ultrahigh sensitivity LC/MS determinations of PGs, TXs, and amino acids because current API-based ionization techniques cannot compete with GC/ECNCI/MS for sensitivity and specificity. Limits of detection were determined for $PGF_{1\alpha}$, 11-dehydro-TXB_2, and phenylalanine as representatives of the three different classes of biomolecules (Table 1). Intense $[M - PFB]^-$ ions were observed for $PGF_{1\alpha}$-PFB, 11-dehydro-TXB_2-PFB, PA-bis-PFB at *m/z* 355, 367, and 344, respectively. CID and MS/MS analysis was performed on the $[M - PFB]^-$ ions from each analyte. Specific product ions were then chosen for LC/SRM/MS analysis in order to determine the limit of detection for each analyte. Using the transitions *m/z* 355 → 311 for $PGF_{1\alpha}$-PFB and *m/z* 367 → 305 for 11-dehydro-TXB_2-PFB on gradient system 3, limits of detection were 50 fg (140 amol) for $PGF_{1\alpha}$ (Figure 10A; lower) and 200 fg (540 amol) for 11-dehydro-TXB_2 (Figure 10B; lower). Using the SRM transition *m/z* 344 → 296 for phenylalanine-bis-PFB on gradient system 4, a limit of detection of 100 fg (610 amol) was

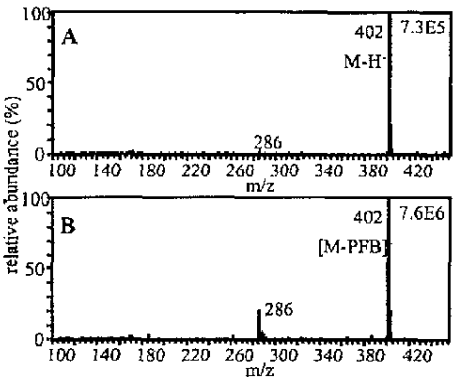

Figure 8. (A) Negative APCI mass spectrum of heptanone ϵ-dGuo. (B) ECAPCI mass spectrum of heptanone ϵ-dGuo-PFB.

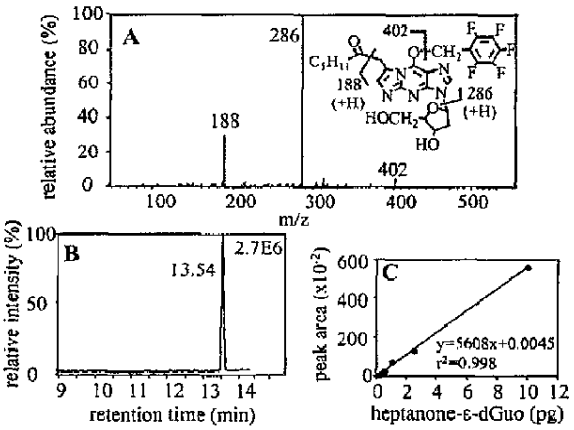

Figure 9. (A) CID and MS/MS analysis of the intense $[M - PFB]^-$ ion *m/z* 402 from the substituted ϵ-Guo-PFB isolated from the reaction of 4-oxononenal with Guo. (B) Total ion current chromatogram from LC/ECAPCI/MS analysis of heptanone-ϵ-dGuo-PFB (10 ng). LC was performed using chromatography system 5. (C) Standard curve for the analysis of heptanone ϵ-dGuo-PFB using LC/ECAPCI/SRM/MS monitoring *m/z* 402 → 286 in the range of 100 fg to 10 pg.

(21) Chaudhary, A. K.; Nokubo, M.; Marnett, L. J.; Blair, I. A. *Biol. Mass Spectrom.* **1994**, *23*, 457–64.
(22) Chaudhary, A. K.; Nokubo, M.; Reddy, G. R.; Yeola, S. N.; Morrow, J. D.; Blair, I. A.; Marnett, L. J. *Science* **1994**, *265*, 1580–2.

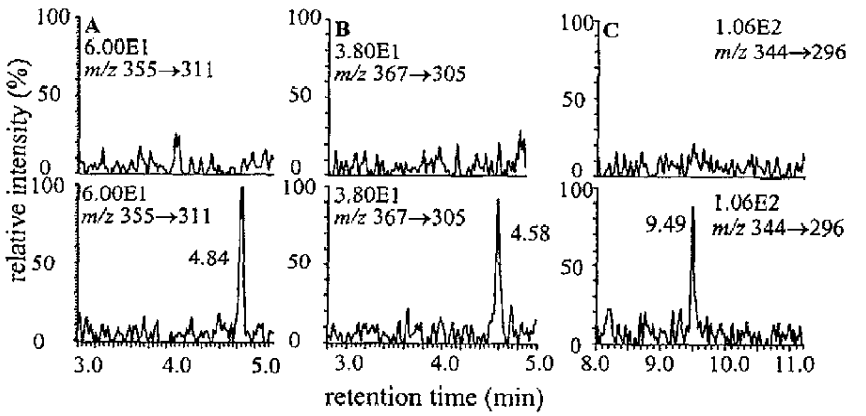

Figure 10. LC/ECAPCI/SRM/MS analysis for the limit of detection of a representative prostaglandin, thromboxane, and amino acid. (A) Top panel: 10 μL of blank solvent injection. Bottom panel: 50 fg (140 amol) of prostaglandin $PGF_{1\alpha}$ on column monitoring the transition *m/z* 355→ 311 at collision energy of 25 eV using chromatography system 3. (B) Top panel: 10 μL of solvent blank. Bottom panel: 200 fg (540 amol) of 11-dehydrothromboxane B_2 on column monitoring the transition *m/z* 367→ 305 at collision energy of 21 eV using chromatography system 3 (C) Top panel: 10 μL of solvent blank. Bottom panel: 100 fg (610 amol) of phenylalanine-mono-PFB monitoring the transition *m/z* 344→ 296 at collision energy of 17 eV using chromatography system 4.

obtained for phenylalanine (Figure 10C; lower). Before each injection, a blank sample was injected in order to show that there was no interference at the retention time of each particular analyte (Figure 10A–C; upper). A linear response for each of these analytes was observed over 4 orders of magnitude (data not shown).

Optimization of LC/ECAPCI/MS Sensitivity. No modifications were made to the commercial APCI source. Attomole sensitivity was attained with a number of biomolecules and drugs using standard conditions and flow rates as noted below. LC flow rates of up to 1 mL/min were obtained with reversed-phase solvents that contained 60–100% methanol. The methanol content of the LC mobile phase was readily adjusted by the use of postcolumn solvent addition. For normal-phase solvents, flow rates of up to 0.3 mL/min were used without any reduction in response. Substitution of air, oxygen, or inert gases such as helium and argon for the nitrogen sheath gas caused a substantial reduction or elimination of the signal. The position of the corona discharge needle was not optimized because these studies were performed using a commercial APCI source. Therefore, it is conceivable that further increases in sensitivity may be possible as a result of improved source design. The corona discharge current was shown to affect sensitivity with an optimal setting of 10–16 μA. Overall improvements in sensitivity for a range of analytes when compared with conventional APCI methodology ranged from 25- to 100-fold (Table 1).

Comparison with GC/MS. Previous GC/ECNCI/MS studies have demonstrated that extremely high sensitivity can be attained with analytes that provide a large cross section for the capture of low-energy electrons.[9] A number of electron capturing derivatives have been reported for the analysis of biomolecules and drugs including trifluoroacetyl (melatonin),[23] PFB [barbiturates, DNA adducts, prostaglandins (PGs), leukotrienes (LTs), epoxyeicosatrienoic acids (EETs), isoprostanes],[10,12,24,25] pentafluorobenzoyl (platelet activating factor),[26] bis(trifluoromethylbenzyl) [thromboxanes (TXs) and PGs],[27] heptafluorobutyryl (aromatic amines),[28] pentafluorodimethylsilyl (sterols),[29] pentafluorophenyl (protein adducts),[30] pentafluorobenzyl-1,2,4-triazoline-3,5-dione (vitamin D analogues),[31] and pentafluorobenzyloxycarbonyl (amino acids),[32] and many of them have been used for ultrahigh sensitivity GC/ECNCI/MS analyses.[10,12,21–25,27,30,32] PFB derivatives have found the widest application in GC/MS studies because they are simple to prepare and they confer excellent electron-capturing properties to the analyte.[4,11,12] This derivative was also found to undergo efficient dissociative electron capture in the APCI source.

An attractive feature of the LC/ECAPCI/MS method is that thermally labile and/or polar molecules can be readily analyzed with high sensitivity. This is generally not possible with GC/MS. For example, it is impossible to distinguish DNA adducts from RNA adducts in the urine as it is necessary to remove the 2′-deoxyribose sugar before analysis by GC/MS.[33] LC/ECAPCI/MS analyses can be readily performed with high sensitivity and specificity for both DNA and RNA adducts without removal of the sugar moiety. Therefore, high-sensitivity analyses of intact urinary DNA adducts will now be possible for the first time using this new technique. Another major advantage of the LC/MS method is the capacity of LC to tolerate excess derivatization reagents when compared with a GC. Furthermore, when multiple

(23) Lewy, A. J.; Markey, S. P. *Science* **1978**, *201*, 741–3.
(24) Prakash, C.; Adedoyin, A.; Wilkinson, G. R.; Blair, I. A. *Biol. Mass Spectrom.* **1991**, *20*, 559–64.
(25) Waugh, R. J.; Morrow, J. D.; Roberts, L. J., II; Murphy, R. C. *Free Radical Biol. Med.* **1997**, *23*, 943–54.
(26) Ramesha, C. S.; Pickett, W. C. *Biomed. Environ. Mass Spectrom.* **1986**, *13*, 107–11.
(27) Barrow, S. E.; Ward, P. S.; Sleightholm, M. A.; Ritter, J. M.; Dollery, C. T. *Biochim. Biophys. Acta* **1989**, *993*, 121–7.
(28) Longo, M.; Cavallaro, A. *J. Chromatogr., A* **1996**, *753*, 91–100.
(29) Jayasinghe, L. Y.; Marriott, P. J.; Carpenter, P. D.; Nichols, P. D. *J. Chromatogr., A* **1998**, *809*, 109–20.
(30) Ranasinghe, A.; Scheller, N.; Wu, K. Y.; Upton, P. B.; Swenberg, J. A. *Chem. Res. Toxicol.* **1998**, *11*, 520–6.
(31) Wang, K.; Davis, P. P.; Crews, T.; Crabriel, L.; Edom, R. W. *Anal. Biochem.* **1996**, *243*, 28–40.
(32) Simpson, J. T.; Torok, D. S.; Girard, J. E.; Markey, S. P. *Anal. Biochem.* **1996**, *233*, 58–66.
(33) Shuker, D. E. G.; Farmer, P. B. *Chem. Res. Toxicol.* **1992**, *5*, 450–60.

biological samples are analyzed, injector fouling is often observed in GC/MS analyses, which can cause a decrease in sensitivity and subsequent loss of analytical data. This is not a problem for LC/MS-based assays.

CONCLUSIONS

Attomole sensitivity was obtained for the analysis of pentafluorbenzyl derivatives of steroids, PGs, TXs, amino acids, and DNA adducts. We anticipate that this ionization technique, which we have termed LC/ECAPCI/MS, will rapidly displace GC/MS for the trace analysis of a wide range of other biomolecules. High-sensitivity analyses will be possible for lipoxins, EETs, hydroxyeicosatetraenoic acids, LTs, isoprostanes, lipids, folates, peptides, and protein adducts. It will also impact significantly on methodology for the trace analysis of drugs such barbiturates, valproate, methotrexate, and 6-mercaptopurine. The sensitivity of LC/ECAPCI/MS under normal-phase conditions using hydrophobic solvents such as hexane is similar to (or better than) that obtained under reversed-phase conditions. This will allow additional flexibility in the trace analysis of biomolecules and drugs because chromatography can be performed on reversed-phase columns, conventional silica columns, multiple silica columns in series, or normal-phase chiral columns. Finally, LC/ECAPCI/MS will confer enhanced specificity for biomolecule analysis because it will be possible to develop assays for several electron-capturing derivatives that have different LC retention times.

ACKNOWLEDGMENT

The authors gratefully acknowledge financial support from the National Institutes of Health in the form of an RO1 grant to I.A.B. (CA65878).

Received for review March 29, 2000. Accepted May 22, 2000.

AC000374A